Small Interference RNA Mediated Silencing of Begomovirus Gene in Commercially Valuable Plants

A
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INTRODUCTION:

Virus diseases are serious constraints to the wide range of crop productivity and profitability. New species of viruses becoming an increasing menace to many crops. Geminiviruses together with Potyviruses are among most devastating plant pathogens and forms the two most important and major plant virus families *Geminiviridae* and *Potyviridae* respectively. Geminiviruses have caused immense losses in many crops and threatened crop production mainly in tropical and subtropical regions (Gibbs and Ohshims, 2010; Scholthof *et al.*, 2011).

Genome of geminiviruses consist of one (monopartite) or two (bipartite) circular ssDNA, packed in icosahedral twinned particles (Böttcher *et al.*, 2004; Krupovic *et al.*, 2009). These viruses infect both monocot (monocotyledonous) as well as dicot (dicotyledonous) plants (Gutierrez, 2000). *Geminiviridae* family currently containing 9 generas named as Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus, Turncurtovirus. Among all the generas Begomovirus is the largest genus with >320 species members (ICTV 10th Report, 2017; Varsani A. *et al.*, 2017). Transmission of Begomovirus takes place by vector whitefly (*Bemisiatabaci*).

Begomoviruses originating from the New World (NW) typically have genomes consisting of two separately encapsidated genomic components, known as DNA-A and DNA-B (Brown *et al.*, 2012). The majority of OW begomoviruses are monopartite in nature, with some bipartite members (Zhang & Ling, 2011).

The common region (CR) present in both genomic components of begomovirus is ~200 nucleotide long segment of intergenic region (IR). The genomes of monopartite (and DNA-A components of bipartite) begomoviruses are typically ~2.7- 3.0 kb in length and have genes in both orientations from a non-coding intergenic region (IR), which contains the promoter elements and the ori of virion-strand DNA replication (Fauquet *et al.*, 2008). The virion strand ori consists of a hairpin structure containing a conserved nonanucleotide (TAATATTAC) sequence in the loop and repeated upstream motifs known as "iterons". The DNA-A component contains six ORFs in both orientations that encode proteins which play major roles in symptom development, viral replication & infectivity.

Coat Protein (CP; V1): Required for encapsidation, insect transmission and movement in plants (Sharma and Ikegami, 2011).

Pre-Coat Protein (Pre-CP; V2): Responsible for virus movement in plants and also known as pathogenicity determinant (Rojas *et al.*, 2005). It also acts as a suppressor of RNA silencing pathway in host (Yadava *et al.*, 2010).

Replication-Initiator Protein (Rep; C1): The one and only virus-encoded gene product required for viral DNA replication (Nash *et al.*, 2011).

Transcriptional activator protein (TrAP; C2): Acts as a suppressor of RNA silencing in bipartite (Yang *et al.*, 2007) and monopartite begomoviruses (Gopal *et al.*, 2007).

Replication enhancer protein (REn; C3): REn is involved in the establishment of an environment which is suitable for virus replication (Pedersen & Hanley- Bowdoin, 1994).

C4 protein (C4): The function of C4 is still unknown but for some viruses it has been shown as a pathogenicity determinant and a suppressor of PTGS by binding of siRNAs (Vanitharani *et al.*, 2004).

The DNA-B component contains two ORFs in opposite orientations encoding:

Nuclear shuttle protein (NSP; BV1): Responsible for transport of viral DNA from the nucleus into the cytoplasm (Sanderfoot *et al.*, 1996).

Movement protein (MP; BC1): Responsible for the movement of viral DNA across plasmodesmatal boundaries (Sanderfoot & Lazarowitz, 1996).

There have been an increasing number of reports of monopartite begomoviruses occurring in association with circular ssDNA satellites, called as alphasatellites (Briddon *et al.*, 2004) and betasatellites (Briddon *et al.*, 2008; Yang X. *et al.*, 2012). Alphasatellites encode a Rep and are capable of autonomous replication in host plant cells (Saunders and Stanley, 1999). Betasatellites, which encode the βC1 protein, depend on the helper virus for replication (Cui *et al.*, 2005).

The betasatellites are very diverse in nature and are associated with helper begomoviruses. Betasatellites contain a region (~100 nt) known as the satellite conserved region (SCR), which is highly conserved between all betasatellites and contains the stem loop. In addition, the betasatellites contain an adenine rich region (A-rich) and a single ORF (the βC1 gene) in the complementary orientation of the molecule. The precise function of betasatellites and its βC1 protein in pathogenesis of viruses although it has been proposed that DNA β may play a direct or an indirect role in replication, facilitating movement, or countering host defence response.

Review of Literature:

Discovery of plant viruses was over a century ago when the science of virology was born. Plant viruses are obligate intracellular parasite on their host. Through their life cycle, from virus accumulation to intracellular, local, and systemic movement, viruses utilize plant proteins, normally involved in host-specific activities, for their own purposes (Richard S. Nelson, 2005).

Viral infection is one of the major biotic stresses on plant which affects the metabolisms as well as the genome of the plant, particularly its expression in terms of transcriptome and proteome. Plant viruses enter cells only through wounds made mechanically or by vectors or by deposition into an ovule by an infected pollen grain (Diener *et al.*, 1993).

Geminiviruses belong to the family *Geminiviridae,* which is the largest plant virus family. Begomoviruses are the largest group among nine generas of family *Geminivirdeae.* They infect a broad range of plants including crops, ornamental and weed plants all over the world. Begomoviruses are divided into two groups Old World and New World viruses according their origin. More than 133 begomovirus species have been characterized comprising of monopartite genomes and all originate from the Old World (OW). No monopartite begomoviruses natives to the New World (NW) have been identified (Polston *et al.*, 1999). A major portion of monopartite begomoviruses have been found to be associated with ssDNA satellites known as betasatellites which are totally dependent on their helper viruses for replication, movement and encapsidation in plants and transmission between plants (Briddon and Stanley, 2006). Further study on the evolution of *begomoviruses* has revealed that the virus-betasatellite complex is also associated with a class of ssDNA components known as alphasatellites (Briddon and Stanley, 2006). Despite having a mechanism to maintain the integrity of their split genomes, component exchange, referred to as pseudo-recombination for begomoviruses, occurs frequently (Pita *et al.,* 2001).

There is a greater genetic variation of DNA-B components in comparison to the DNA-A components. Although the vast majority of NW and some OW *begomoviruses* show near perfect co-evolution of the DNA-A and DNA-B components, this is not the case for the majority of OW viruses (Briddon *et al.*, 2010).

Begomoviruses require only DNA-A for systemic infection (Dry *et al.,* 1999). The CP gene is the most highly conserved gene in the family *geminiviridae* (Wyatt, 1996). CP is the sole structural protein of geminiviruses and has been shown to play a determinative role in the transmission of these viruses. This gene sequence efficiently predicts discrete strains, species, and taxonomic lineages of *begomoviruses*, which has been accepted by ICTV as a desirable marker for virus identity when a full length genomic sequence is not available (Mayo, 1997). The whitefly-transmitted geminiviruses are in the genus Begomovirus.

They comprise the largest number of species (288 of 325 total species) in the family *geminiviridae* (Brown *et al.*, 2012). The remarkable emergence of these viruses has been driven by its vector, *B. tabaci*, especially the polyphagous biotype B that has spread throughout the world (Gilbetson *et al.*, 2015). Begomoviruses can be subdivided into two major groups: bipartite viruses, those with a genome composed of two ~2.6 kb DNA components (referred to as DNA-A and DNA-B), which are prevalent in the New World (NW, e.g., southern states of the United States of America (USA), Mexico, the Caribbean and South America); and monopartite viruses, those with a genome composed of a single ~2.9 kb genomic DNA, which are prevalent in the Old World (OW, e.g., Europe, Africa, Asia and Australia). Many monopartite begomoviruses are associated with satellite DNAs (alphasatellites and betasatellites), some of which enhance pathogenicity (Zhou, 2013).

The vector of begomoviruses, *B. tabaci* is a complex of morphologically similar, but biologically and genetically distinct populations. The species *B. tabaci* is divided into biotypes (e.g., A, B, Q), which are probably distinct enough to be considered as species (de Barro *et al.*, 2011).

Geminivirus infection triggers the process of RNA silencing in the host (Akbergenov, R. *et al.*, 2006). The natural antivairal defence mechanism which plays important role in genome maintenance and plant development is Post-Transcriptional Gene Silencing (PTGS) (Saxena S. *et al.*, 2013). The effector molecules of RNA silencing pathway are small RNAs that regulate gene expression in a sequence-dependent manner. The main classes of sRNAs are small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Vazquez, F *et al.*, 2010).

RNAi has revolutionized the possibilities for creating custom ''knock-downs'' of gene activity. RNAi operates in both plants and animals, and uses double stranded RNA (dsRNA)

as a trigger that targets homologous mRNAs for degradation or inhibiting its transcription or translation (Saxena S. *et al.*, 2011).

In plants, RNA silencing-based antiviral defense is mediated by Dicer-like (DCL) proteins producing short interfering (si) RNAs. *Arabidopsis* encodes five double-stranded RNA binding (DRB) proteins. DRB1 and DRB2 are involved in microRNA (miRNA) biogenesis, while DRB4 functions in cytoplasmic posttranscriptional small interfering RNA (siRNA) pathways. DRB3 and DRB5 are not involved in double-stranded RNA (dsRNA) processing but assist in silencing transcripts targeted by DRB2-associated miRNAs (Raja, P. *et al.*, 2014). Generally, 21–22 nt siRNAs target viral coding regions, whereas 24 nt siRNAs were found to be targeting IR of the viral genome (Akbergenov, R. *et al.*, 2006). In plants and some animals, RNA-dependent RNA polymerase (RDR) generates precursors of secondary sRNAs that reinforce silencing. Geminiviruses are inducers of RNA silencing owing to the bidirectional transcription of their genomes (Hanley-Bowdoin, L *et al.*, 2000), aberrant read-through transcription (Shivaprasad, P.V. *et al.*, 2005), and the overlapping ORFs which results in the production of double-stranded RNA (dsRNA)—a key instigator of the RNA silencing pathways (Shunmugiah V. R. *et al.*, 2017).

Micro RNAs are the class of 21-nt-length small non-coding RNAs (ncRNAs) that function ascrucial effector molecules of PTGS in plants. Although the antiviral nature of host-derived miRNAs is abundant in animal–virus interactions, few instances of plant miRNA-mediated antiviral activities are also documented (Shunmugiah V. R. *et al.*, 2017). The expression of viral derived miRNAs has been demonstrated during the infection of African cassava mosaic virus and East African cassava mosaicvirus—Uganda (Maghuly, F. *et al.*, 2014).

Objectives:

- Molecular characterization and sequencing of begomoviral genome.
- Intra specific recombination study of begomovirus by using various available data tools.
- Detection of small interfering RNA sequences and preparation of constructs containing designed siRNA capable of inducing virus resistance in transgenic plants.
- Protein expression and *in silico* structural analysis of proteins involved in gene silencing.

Materials and Methods:

Collection of plant samples:

The infected leaf samples were photographed with a high resolution camera from the different regions of North India on the basis of typical begomoviruses symptoms i.e., yellowing of leaf veins, yellow mosaics patterns, curling of fractional or complete leaves, leaf distortion, chlorosis and decrease in leaf size and stunting in plants and stored at -20°C until utilized.

Genomic DNA extraction:

Total DNA from leaf samples was extracted by using a CTAB (Cetyl Tri-methyl Ammonium Bromide) method (Manen *et al.*, 2005). The DNA pellet was washed with cold 70% ethanol twice, vacuum dried, and resuspended in 100 µl of TE buffer.

Estimation of DNA quantity and quality:

Concentration of DNA was estimated through Nanodrop (Cuvette free spectrophotometer) to check the quantity of DNA/µl for further study. DNA quality was also checked by running the DNA samples on 0.6 % agarose gel using 1X TAE. The gel was stained with ethidium bromide (0.3 mg per ml) and read under UV light on a transilluminater or Gel-doc system.

Screening by PCR:

For amplification of DNA by PCR a reaction mixture of 50µl containing 10pg- 1µg template DNA, 5µl 10X Taq polymerase buffer, 5µl 2mM dNTPs, 1.5mM MgCl2, 0.5µM of each primer and 1.25 units Taq DNA polymerase will prepared in a 0.25ml thin walled PCR tube. The reaction mixture was carried out in thermal cycler, using begomovirus specific universal primers against coat protein (CP) and β-satellite. This confirmed the molecular level infection of the begomovirus in plant samples.

Rolling Circle Amplification (RCA) and Restriction digestion:

Full length sequences of DNA-A and betasatellite was obtained by rolling circle amplification of CP positive samples using Templiphi kit (GE Healthcare) followed by restriction digestion with different restriction enzymes.

Cloning and Sequencing:

The positive RCA digested product will be cloned into pGEM-T easy vector (Promega, Madison, Wisconsin, USA) following the manufacturer's instructions. Plasmids will be introduced by transformation into *Escherichia coli* DH_5 alpha. The cloned viral inserts sequenced will be sequences by Sanger's method.

Sequence analysis and Phylogeny:

Sequence obtained will be translated using EXPASY (Expert Protein Analysis System) tool. Nucleotide and protein sequence data will analyse using BLAST program at NCBI then Clustal W will be performed for multiple sequence alignment of the test sequence with other begomoviral genomes available at NCBI Database. Phylogenetic tree (NJ tree & ML tree) will be formed by using MEGA 7.0 software (www.megasoftware.net).

Recombination analysis:

Recombination analysis will be performed by using RDP3 (Martin *et al.*, 2010).

Designing of siRNA:

By using begomoviral genome as input query siRNA will be designed in programs like DEQOR (Henschel *et al.*, 2004), Jemboss software (http://emboss.sourceforge.net/jemboss/). These tools show all the possible siRNA for each gene with GC content and thermodynamic value.

Transgenic plant:

Suitable and desirable siRNA with the help of suitable vector will be introduced in plants. And further plant will be checked for resistance development.

Screening of Transformed plants using PCR:

Again plant DNA will be extracted from transgenic plants and transgene will be checked by performing PCR and agarose gel electrophoresis.

Significance of Work:

The major problems faced in the field of Agriculture are loss in crop yield caused by insects, Herbs (weeds), viruses and the pathogens and the pests associated loss is about 14% of total agricultural production (Santha *et al.,* 2012). A key component to the control of plant virus disease is availability of diagnostic tool for rapid and accurate identification of virus causing disease. PCR based detection of virus is the best and most accurate tool for complete identification of begomovirus, opportunities are now arising to design degenerate PCR primers for detection of viruses on genus level. The use of degenerate primers has not only facilitated the rapid detection of many begomoviruses but has also enabled partial genomic sequencing for taxonomic purposes. The phylogenetic analysis based on nucleotide sequences based on coat protein and movement protein gene will suggest ancestral groups of different begomoviruses.

The use of pesticides has resulted in adverse effects on the beneficial organisms and other plant parts such as leaves, fruits and has reached pollution levels, which has become a major concern for environmentalists. Therefore, products of crops resistant to insects have been the first priority in crop biotechnology. Genetic transformation has lead the possibility of transforming crops for enhanced resistance to insects through the use of pathogen derived resistance.

The knowledge of the 3D structure of a protein is an extremely useful prerequisite for the understanding of the function and for the rational modification of proteins. *In silico* study of protein, nucleic acid helpful in almost all research fields, it not only saves money but also saves our valuable time. Homology Modelling is becoming a useful technique in the field of bioinformatics because the knowledge of the three-dimensional structure of a protein would be an invaluable aid to understand the details of a particular protein.

Future Prospects:

Diseases caused by begomoviral infection have become a significant limiting factor in the sustainable production of vegetables and crops as well as significant financial lost throughout the INDIA. Therefore improved scientific understanding about viruses means virus identification and its characterization is required for development of successful crop management strategies. This project include Sequencing of virus genome, phylogenetic analysis and its Insilco analysis that will help in designing reliable management strategies against begomovirus and would increase our knowledge on the variability and evolution of this group of viruses and also enhance information about natural host of begomovirus. Single and mixed infections by begomoviruses and their recombinants are probably responsible for new epidemics in several crops and in ornamental plants.

In future we can use transgenic plants against begomovirus infection commercially which may enhance crop yield as well as quality. The future challenge for scientists in this field is to develop strategies that broaden the breadth and increase the degree of resistance. This will be achieved through fundamental studies that elucidate the molecular mechanisms of resistance and apply the knowledge thus derived to develop second and third generation resistance genes with increased efficacy.

Northern India climatic conditions support the high population of *Bemisiatabaci* (whitefly). This study will give the information about the natural host plants of begomoviruses and beta satellites in Northern states.

Recombination analysis will help in revealing possible emergence of new begomoviruses complexes. Co-inoculation of begomoviruses and different beta satellites will help in knowing the role of beta satellites in disease symptom induction and exchange of beta satellites among different host plant infecting begomoviruses.

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